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<b>TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371</b>				017753-137	
				U.S. APPLICATION NO. (if known, see 37 C.F.R. 1.5) unassigned 09/762573	
INTERNATIONAL APPLICATION NO. PCT/FR00/01559		INTERNATIONAL FILING DATE 7 June 2000		PRIORITY DATE CLAIMED 8 June 1999	
TITLE OF INVENTION COMPOSITION FOR IMPLEMENTING A CYTOTOXIC, IN PARTICULAR AN ANTITUMORAL OR ANTIVIRAL, TREATMENT IN A MAMMAL					
APPLICANT(S) FOR DO/EO/US Etienne REGULIER; Philippe ERBS					
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:					
1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. 371. 2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. 371. 3. <input checked="" type="checkbox"/> This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and the PCT Articles 22 and 39(1). 4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. 5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. 371(c)(2)) a. <input checked="" type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau. c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US). 6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. 371(c)(2)). 7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3)) a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau). b. <input type="checkbox"/> have been transmitted by the International Bureau. c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired. d. <input type="checkbox"/> have not been made and will not be made. 8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)). 9. <input checked="" type="checkbox"/> An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). 10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)). Items 11. to 16. below concern other document(s) or information included: 11. <input type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. 12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. 13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment. 14. <input type="checkbox"/> A substitute specification. 15. <input type="checkbox"/> A change of power of attorney and/or address letter. 16. <input type="checkbox"/> Other items or information:					

17. ☒ The following fees are submitted:

CALCULATIONS

PTO USE ONLY

**Basic National Fee (37 CFR 1.492(a)(1)-(5)):**

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO ..... \$1,000.00 (960)

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO ..... \$860.00 (970)

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... \$710.00 (958)

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) ..... \$690.00 (956)

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) ..... \$100.00 (962)

**ENTER APPROPRIATE BASIC FEE AMOUNT =**

\$ 860.00

Surcharge of **\$130.00 (154)** for furnishing the oath or declaration later than months from the earliest claimed priority date (37 CFR 1.492(e)).

20 ☐ 30 ☐

\$

Claims	Number Filed	Number Extra	Rate		
Total Claims	23 -20 =	3	\$18.00 (966)	\$	54.00
Independent Claims	2 -3 =	0	\$80.00 (964)	\$	0
Multiple dependent claim(s) (if applicable)				+ \$270.00 (968)	\$

**TOTAL OF ABOVE CALCULATIONS =**

\$ 914.00

Reduction for 1/2 for filing by small entity, if applicable (see below).

\$

-

**SUBTOTAL =**

\$ 914.00

Processing fee of **\$130.00 (156)** for furnishing the English translation later than months from the earliest claimed priority date (37 CFR 1.492(f)).

20 ☐ 30 ☐

\$

+

**TOTAL NATIONAL FEE =**

\$ 914.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **\$40.00 (581)** per property +

\$ 40.00

**TOTAL FEES ENCLOSED =**

\$ 954.00

Amount to be:  
refunded \$

charged \$

- a. ☐ Small entity status is hereby claimed.
- b. ☒ A check in the amount of \$ 954.00 to cover the above fees is enclosed.
- c. ☐ Please charge my Deposit Account No. 02-4800 in the amount of \$ \_\_\_\_\_ to cover the above fees. A duplicate copy of this sheet is enclosed.
- d. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 02-4800. A duplicate copy of this sheet is enclosed.

**NOTE:** Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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30,427

REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of )  
Etienne REGULIER et al. ) Group Art Unit: Unassigned  
Application No.: Unassigned ) Examiner: Unassigned  
Corresponds to PCT/FR00/01559 )  
International Filing Date: 7 June 2000 )  
For: COMPOSITION FOR )  
IMPLEMENTING A CYTOTOXIC, IN )  
PARTICULAR AN ANTITUMORAL )  
OR ANTIVIRAL, TREATMENT IN A )  
MAMMAL )

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents  
Washington, D.C. 20231

Sir:

Prior to examination, please amend the above-captioned application as follows:

IN THE CLAIMS:

Kindly amend the claims as follows:

Claim 2, lines 1-2, change "characterized in that" to --wherein--;

lines 2-4, delete ", and more particularly selected from the group  
consisting of the MIP1 $\alpha$  and MIP1 $\beta$  chemokines".

3. (Amended) Composition according to [either of Claims 1 and 2] Claim 1,  
[characterized in that] wherein said polypeptide having cytotoxic activity is [chosen]  
selected from the group consisting of cytokines, proteins encoded by suicide genes and anti-  
angiogenic protein factors.

4. (Amended) Composition according to Claim 3, [characterized in that] wherein said polypeptide having cytotoxic activity is a cytokine [chosen] selected from the group consisting of interferons  $\alpha$ ,  $\beta$  and  $\gamma$ , interleukins, tumor necrosis factors and colony stimulating factors.

Claim 5, lines 1-2, change "characterized in that" to --wherein--.

Claim 6, lines 1-2, change "characterized in that" to --wherein--.

Claim 7, line 1, change "one of Claims 1 to 6" to --Claim 1--;  
line 2, change "characterized in that it comprises" to --comprising--.

Claim 8, lines 1-2, change "characterized in that" to --wherein--;  
line 3, after "from" insert --the group consisting of--.

Claim 9, lines 1-2, change "characterized in that" to --wherein--.

10. (Amended) Composition according to Claim 4, [characterized in that] wherein said polypeptide having cytotoxic activity is an anti-angiogenic protein factor [chosen] selected from the group consisting of angiostatin, endostatin, platelet factor PF4, thrombospondin-1, PRP, VEGF, metalloproteases and urokinase.

Claim 11, lines 1-2, change "characterized in that" to --wherein--.

Claim 12, line 2, change "characterized in that" to --wherein--.

Claim 13, line 2, change "characterized in that" to --wherein--.

Claim 15, lines 1-2, change "characterized in that it" to --wherein said vector--.

17. (Amended) Method for preparing a viral particle according to Claim 16,  
[according to which] wherein:

(i) introducing a viral vector according to claim 15 [is introduced] into a cell  
capable of producing said vector, so as to obtain a transfected cell,

(ii) culturing said transfected cell [is cultured] under suitable conditions in order  
to allow the production of said viral particle, and

(iii) recovering said viral particle [is recovered] from the cell culture.

Claim 18, line 7, change "Claims 1 to 10" to --Claim 1--.

Claim 19, lines 2-3. change "characterized in that it comprises" to  
--comprising--;

line 3, delete "one of Claims 1";

lines 4-5, delete in their entirety;

line 6, delete "according to according to" and change "18" to --1--;

line 7, change "acceptable from a pharmaceutical point of view" to  
--pharmaceutically acceptable--.

Claim 20, lines 2-3, delete in their entirety;

line 4, delete "prodrug" and insert therefor --comprising--.

Claim 21, line 2, change "characterized in that" to --wherein-- and delete  
"selected from";

line 3, change "and" to --or--.

Kindly cancel claim 22 without prejudice or disclaimer.

Kindly add the following claims:

--23. The composition according to Claim 2, wherein said MIP1 chemokine is  
MIP1 $\alpha$  or MIP1 $\beta$ .

24. A method for treating a patient in need of a cytotoxic agent, said method  
comprising administering an effective amount of the composition of Claim 1 to a patient in  
need of such treatment.--

#### **REMARKS**

Entry of the foregoing amendments are respectfully requested.

Should the Examiner have any questions concerning the subject application, a telephone call to the undersigned would be appreciated.

Respectfully submitted,

BURNS, DOANE, SWICKER & MATHIS, L.L.P.

By: 

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Date: February 8, 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No. :

U.S. National Serial No. :

Filed :

PCT International Application No. : PCT/FR00/01559

VERIFICATION OF A TRANSLATION

I, the below named translator, hereby declare that:

My name and post office address are as stated below;

That I am knowledgeable in the French language in which the below identified international application was filed, and that, to the best of my knowledge and belief, the English translation of the international application No. PCT/FR00/01559 is a true and complete translation of the above identified international application as filed.

I hereby declare that all the statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the patent application issued thereon.

Date: January 25, 2001

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As printed

(54) Title: COMPOSITION FOR IMPLEMENTING A CYTOTOXIC, IN PARTICULAR AN ANTITUMORAL OR ANTIVIRAL,  
TREATMENT IN A MAMMAL(54) Titre: COMPOSITION DESTINEE A LA MISE EN OEUVRE D'UN TRAITEMENT CYTOTOXIQUE, NOTAMMENT  
ANTITUMORAL OU ANTIVIRAL, CHEZ UN MAMMIFERE

(57) Abstract: The invention concerns a composition for implementing a cytotoxic treatment in a mammal comprising: (i) a nucleic acid sequence coding for all or part of an MIP chemokine; (ii) at least a nucleic acid sequence coding for all or part of a polypeptide having at least a cytotoxic activity, said nucleic acid sequences being placed under the control of elements required for their expression in said mammal's host cell.

(57) Abrégé: La présente invention concerne une composition destinée à la mise en oeuvre d'un traitement cytotoxique chez un mammifère comprenant: (i) une séquence d'acide nucléique codant pour tout ou partie d'une chimiokine MIP, (ii) au moins une séquence d'acide nucléique codant pour tout ou partie d'un polypeptide ayant au moins une activité cytotoxique; lesdites séquences d'acide nucléique étant placées sous le contrôle des éléments nécessaires à leur expression dans une cellule hôte dudit mammifère.

WO 00/74629 A2

PCT/FR00/01559

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WO 00/74629

PCT/FR00/01559

**Composition intended for the implementation of a  
cytotoxic, in particular antitumor or antiviral,  
treatment in mammals**

5 The present invention relates to a cytotoxic  
composition comprising a first nucleic acid sequence  
encoding all or part of an MIP chemokine, and a second  
nucleic acid sequence encoding all or part of a  
polypeptide having at least cytotoxic, in particular  
10 antitumor or antiviral, activity. The present invention  
is particularly useful in the context of the  
implementation of a treatment for proliferative or  
infectious diseases by gene therapy.

To date, the most encouraging results obtained  
15 in the context of antitumor treatments concern combined  
treatments associating treatment based on chemical  
compounds (chemotherapy) and treatment based on the use  
of radiation (radiotherapy). Besides the considerable  
inconveniences that this type of treatment causes the  
20 patient, it is noted, in a large number of cases, that  
tumor cells, which may or may not be of metastatic  
type, persist in the individual treated, possibly  
causing a relapse and therefore not allowing complete  
remission.

Recent studies carried out in the cancer field  
25 have proposed adapting gene therapy protocols to  
antitumor therapy. In this respect, mention may be  
made, for example, of the studies by Meneguzzi et al.,  
1991, Virology, 181, 61-69, relating to immunization  
30 against tumor cells using a vaccinia recombinant vector  
expressing the E6 and E7 genes of the human type 16  
papilloma virus. Mention may also be made of the  
content of French patent FR 92/03120, relating to the  
use of a recombinant adenovirus expressing a cytokine  
35 in the context of an antitumor gene therapy.

Cytokines are molecules which are naturally  
produced subsequent to an antigenic stimulation or to  
an inflammatory reaction (Gillis and Williams, 1998,

Curr. Opin. Immunol., 10, 501-503) and which have been shown to be useful in the context of the treatment of certain cancers, in particular by Oettger (Curr. Opin. Immunol., 1991, 3, 699-705). Thus, Leroy et al. (1998, 5 Res.Immunol. 149 (7-8): 681-684) have shown that the production of cytokines at the sites of the tumor, after intratumoral administration of recombinant vectors, allows the induction of an immune response associated with inhibition of tumor growth. However, 10 this antitumor response, though encouraging, does not enable the definitive disappearance of the tumor cells to be obtained and, consequently, does not enable the implementation of a satisfactory antitumor treatment.

With regard to chemokines, they constitute a 15 subclass of the cytokine family. They differ from the other cytokines by their chemoattractive property, in particular in the natural processes of chemotaxis and in particular of attraction of the cells of the immune system toward the tissues in which lies the 20 inflammation or the infection, and by their anti-angiogenic properties.

Chemokines are proteins of low molecular weight (between 8 and 10 kd), small in size (from 70 to 80 amino acids), the amino acid sequences of which have a 25 low degree of homology (ranging from 10 to 70% according to the chemokines under consideration), making it possible to define, to date, approximately 50 different chemokines. These chemokines can, however, be subdivided into 4 major families, relating to the 30 position of the cysteine residues that they contain. The  $\alpha$  family, in which the N-terminal end comprises 2 cysteines separated by a single amino acid (chemokines of IL-8, NAP-2, GCP-2 type) and the  $\beta$  family, in which the N-terminal end comprises 2 adjacent cysteines 35 (chemokines of RANTES, MIP1, MCP1 type) are the best characterized (Horuk, R., 1994, Trends Pharmacol. Sci. 15, pages 159-165; Murphy, P.M., 1994, Annu. Rev. Immunol., 12, pages 593-633).

Moreover, the group of Dilloo et al. (1996, Nature Medicine, vol. 2, Number 10, 1090-1095) has shown that the coexpression, after recombinant administration to mice of fibroblasts modified ex vivo using retroviral vectors, of a specific chemokine, lymphotactin (Lptn), and of interleukin-2 (IL2) makes it possible to stimulate the antitumor immune response of the animal treated. However, this effect is limited over time and allows only a transient control of the tumor volume and no remission in the animals treated.

It is therefore desirable to have novel compositions allowing, in particular, the implementation of effective antitumor treatments which are easy to set up, i.e. allowing a sustained control of the tumor volume and an increase in the survival rate of the patients treated.

We have now identified novel cytotoxic compositions in which the various constituents are chosen so as to obtain a synergistic effect of their respective activities and improved properties of said constituents. More particularly, such compositions make it possible to inhibit or to delay cell proliferation by inducing the specific death of the cells, in particular the tumor cells, better antigen presentation and/or stimulation of the immune cells of the host organism. The present invention offers an advantageous and effective alternative to the techniques of the prior art, in particular for treating cancer of humans or of animals.

The invention relates, firstly, to a composition intended for the implementation of a cytotoxic, for example antitumor or antiviral, treatment or any applications requiring cell death, in mammals, comprising:

- (i) a nucleic acid sequence encoding all or part of an MIP chemokine,
- (ii) at least one nucleic acid sequence encoding all or part of a polypeptide having at least cytotoxic activity,

said nucleic acid sequences being placed under the control of the elements required for their expression in a host cell of said mammal.

In the context of the present invention, it is possible to use in (i) all of the nucleic acid sequence encoding the MIP chemokine (for Macrophage Inflammatory Protein) or only a part of this polypeptide, or a derived or mutated polypeptide provided that the function and the properties of the MIP chemokine are conserved. For the purpose of the present invention, the expression "mutation" is intended to mean a deletion and/or a substitution and/or an addition of one or more nucleotides. Similarly, it is conceivable to use a sequence encoding a hybrid chemokine originating from the fusion of the sequences encoding a chemokine of MIP type and of the sequence encoding at least one chemokine of another type (RANTES, MCP 1, etc.).

In the context of the present invention, the preferred MIP chemokine is the chemokine of MIP 1 type, and more particularly selected from the group consisting of the MIP1 $\alpha$  and MIP1 $\beta$  chemokines, the properties of which have been demonstrated by Wolpe et al., 1988, J. Exp. Med, 167, 570-581.

MIP1 $\alpha$ , the nucleic acid and peptides sequences of which are described in Obaru et al., 1986, J. Biochem. 99, 885-894, the content of which is incorporated into the present application by way of reference, is produced by T lymphocytes and monocytes. It enables the chemoattraction of eosinophils and of T lymphocytes during respiratory tract infections; of monocytes and of neutrophils during rheumatoid arthritis, digestive system inflammations or meningitis of bacterial origin. In addition, it inhibits the proliferation of hematopoietic precursors.

MIP1 $\beta$ , the nucleic acid and peptide sequences of which are described in Brown et al., 1989, J. Immunol. 142, 679-68, the content of which is incorporated into the present application by way of

reference, is also produced by T lymphocytes and monocytes. It exerts its chemoattractive properties on monocytes and neutrophils in bone arthritis cases and bacterial meningitis. Like MIP1 $\alpha$ , it inhibits the proliferation of hematopoietic precursors.

Natural variants of said MIP1 $\alpha$  and MIP1 $\beta$  protein exist which are known to those skilled in the art and which bear, for example, the names GOS19, LD78, pAT464, TY5 (from mice) or SIS  $\alpha$  (from mice) for MIP1 $\alpha$ , or pAT744, Act-2, G-26, H-400 (from mice) or hSIS  $\gamma$  (from mice) for MIP1 $\beta$ . In the specific case of MIP1 $\beta$ , the sequence corresponding to Act-2 (Lipes et al., 1988, PNAS, 85, 9704-9708, the content of which is incorporated herein by way of reference) will, for example, be chosen.

The expression "polypeptide having at least cytotoxic activity" is intended to refer to any peptide substance capable of inducing or of activating an immune response directed specifically against a tumor cell (the cytotoxic activity is then called antitumor activity) or a cell infected with a virus (the cytotoxic activity is then called antiviral activity), or of inhibiting the growth and/or the division of such a cell, in particular such a tumor or infected cell. According to one preferred case, said cytotoxic activity causes the death of said cell. According to one specific case, it would also be possible to use compositions according to the present invention in pathological cases associated with cell proliferation, such as for example the phenomena of restenosis.

The chemoattraction activity of a given polypeptide, in particular a polypeptide derived from the MIP chemokine, on cells involved in the immune reactions (such as, for example, eosinophils, T lymphocytes, monocytes or neutrophils) can be evaluated using a chemotaxis assay (Maghazachi, 1993, Nature Immunity, 12, 57). Similarly, since this type of chemokine inhibits the proliferation of hematopoietic precursors, it is possible to evaluate such a property

*in vitro* according to Graham et al., 1992, Growth Factors, 7, 151.

5 The cytotoxic activity of a given polypeptide, in particular an antitumor activity, can be evaluated *in vitro* by measuring cell survival either with short-term viability assays (such as, for example, the tryptan blue or MTT assay) or with clonogenic survival assays (formation of colonies) (Brown and Wouters, 1999, Cancer Research, 59, 1391-1399) or *in vivo* by 10 measuring the growth of tumors (size and/or volume) in an animal model (Ovejera and Houchens, 1981, Semin. Oncol., 8, 386-393).

According to a first variant, the invention relates to a composition characterized in that said 15 polypeptide having cytotoxic activity is chosen from cytokines, proteins encoded by a gene called "suicide gene" and anti-angiogenic protein factors.

More particularly, when said polypeptide in (ii) is a cytokine, it is preferably a cytokine chosen 20 from interferons  $\alpha$ ,  $\beta$  and  $\gamma$ , interleukins, and in particular IL-2, IL-4, IL-6, IL-10 or IL-12, tumor necrosis factors (TNFs) and colony stimulating factors (GM-CSF, C-CSF, M-CSF, etc.).

According to a preferred embodiment, said 25 cytokine is selected from interleukin-2 (IL-2) and interferon gamma (IFN- $\gamma$ ). Interleukin-2 is in particular responsible for the proliferation of activated T-lymphocytes, and for the multiplication and activation of the cells of the immune system (for the 30 nucleic acid sequence see, in particular, FR 85/09480). IFN- $\gamma$  activates phagocytic cells and increases the expression of the class I and II surface antigens of the major histocompatibility complex (for the nucleic acid sequence see, in particular, FR 85/09225). Said 35 nucleic acid sequences are incorporated into the present application by way of reference.

According to another embodiment, the composition according to the invention is characterized in that it comprises in (ii) at least two nucleic acid

sequences encoding all or part of interleukin-2 (IL-2) and all or part of interferon gamma (IFN- $\gamma$ ).

According to a second variant, the invention also relates to such a composition characterized in that said polypeptide in (ii) has at least an enzymatic activity selected from thymidine kinase activity, purine nucleoside phosphorylase activity, guanine or uracil or orotate phosphoribosyl transferase activity and cytosine deaminase activity.

Several studies have made it possible to identify polypeptides which are not toxic as such, but which have catalytic enzymatic properties capable of transforming an inactive substance (prodrug), for example a nucleoside or a nucleoside analog, into a substance which is highly toxic for the cell, for example a modified nucleoside which can be incorporated into the DNA or RNA chains in elongation, with, as a consequence, in particular the inhibition of cell division or cellular dysfunctioning leading to the death of the cell containing such polypeptides. The genes encoding such polypeptides are termed "suicide genes". Many suicide gene/prodrug pairs are currently available. Mention may be made more particularly of the pairs:

- type 1 herpes simplex virus thymidine kinase (HSV-1 TK) and acyclovir or gancyclovir (GCV) (Caruso et al., 1993, Proc. Natl. Acad. Sci. USA 90, 7024-7028; Culver et al., 1992, Science 256, 1550-1552; Ram et al., 1997, Nat. Med. 3, 1354-1361);

- rat cytochrome p450 and cyclophosphamide (Wei et al., 1994, Human Gene Therapy 5, 969-978);

- *Escherichia coli* (*E. coli*) purine nucleoside phosphorylase and 6-methylpurine deoxyribonucleoside (Sorscher et al., 1994, Gene Therapy 1, 233-238);

- *E. coli* guanine phosphoribosyl transferase and 6-thioxanthine (Mzoz and Moolten, 1993, Human Gene Therapy 4, 589-595) and

- cytosine deaminase (CDase) and 5-fluorocytosine (5FC).



More particularly, CDase is an enzyme which is involved in the metabolic pathway of pyrimidines, by which exogenous cytosine is transformed, via hydrolytic deamination, into uracil. CDase activities have been demonstrated in prokaryotes and lower eukaryotes (Jund and Lacroute, 1970, J. Bacteriol. 102, 607-615; Beck et al., 1972, J. Bacteriol. 110, 219-228; De Haan et al., 1972, Antonie van Leeuwenhoek 38, 257-263; Hoeprich et al., 1974, J. Inf. Dis. 130, 112-118; Esders and Lynn, 1985, J. Biol. Chem. 260, 3915-3922), but they are absent in mammals (Koechlin et al., 1966, Biochem Pharmacol. 15, 435-446; Polak et al., 1976, Chemotherapy 22, 137-153). The *Saccharomyces cerevisiae* (*S. cerevisiae*) *FCY1* and *E. coli* *codA* genes encoding, respectively, the CDase of these two organisms are known and their sequences are published (EP 402 108; Erbs et al., 1997, Curr. Genet. 31, 1-6; WO 93/01281).

CDase also deaminates a cytosine analog, 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU), which is a highly cytotoxic compound in particular when it is converted into 5-fluoro-UMP (5-FUMP). Cells lacking CDase activity, due either to an inactivating mutation of the gene encoding the enzyme, or to their natural deficiency for this enzyme (for example, mammalian cells), are resistant to 5-FC (Jund and Lacroute, 1970, J. Bacteriol. 102, 607-615; Kilstrup et al., 1989, J. Bacteriol. 1989 171, 2124-2127). On the other hand, it has been shown that it is possible to transmit the sensitivity to 5-FC to mammalian cells into which the sequence encoding CDase activity has been transferred (Huber et al., 1993, Cancer Res. 53, 4619-4626; Mullen et al., 1992, Proc. Natl. Acad. Sci. USA 89, 33-37; WO 93/01281). In addition, in this case, the neighboring nontransformed cells also become sensitive to 5-FC (Huber et al., 1994, Proc. Natl. Acad. Sci. USA 91, 8302-8306). This phenomenon, called bystander effect, is due to the excretion, by the cells expressing the CDase activity, of 5-FU which intoxicates the neighboring cells by simple diffusion

through the cell membrane. This passive diffusion property of 5-FU constitutes an advantage with respect to the reference system tk/GCV, for which the bystander effect requires a contact with the cells which express tk (Mesnil et al., 1996, Proc. Natl. Acad. Sci. USA 93, 1831-1835). This effect consequently constitutes an additional asset of the use of CDase in the context of gene therapy, in particular anticancer gene therapy.

However, the sensitivity to 5-FC varies a lot according to the cell lines. Poor sensitivity is observed, for example, in PANC-1 (carcinoma of the pancreas) and SK-BR-3 (breast adenocarcinoma) human tumor lines transduced with a retrovirus expressing the *E. coli* *codA* gene (Harris et al., 1994, Gene Therapy 1, 170-175). This undesirable phenomenon might be explained by the absence of or poor endogenous conversion of the 5-FU formed by the enzymatic action of the CDase into cytotoxic 5-FUMP. This step, normally carried out in mammalian cells by orotate phosphoribosyl transferase (Peters et al., 1991, Cancer 68, 1903-1909), may be absent in certain tumors and thus make gene therapy based on CDase ineffective.

In prokaryotes and lower eukaryotes, uracil is transformed into UMP by the action of uracil phosphoribosyl transferase (having, consequently, UPRTase activity). This enzyme also converts 5-FU into 5-FUMP. Thus, *fur1* mutants of the *S. cerevisiae* yeast are resistant to high concentrations of 5-FU (10 mM) and of 5-FC (10 mM) since, in the absence of UPRTase activity, 5-FU, originating from the deamination of 5-FC by CDase, is not transformed into cytotoxic 5-FUMP (Jund and Lacroute, 1970, J. Bacteriol. 102, 607-615). The *upp* and *FUR1* genes encoding the UPRTase of *E. coli* and of *S. cerevisiae*, respectively, have been cloned and sequenced (Andersen et al., 1992, Eur. J. Biochem. 204, 51-56; Kern et al., 1990, Gene 88, 149-157).

For the purpose of the present invention, a polypeptide having UPRTase activity refers to a polypeptide capable of converting uracil, or one of its

derivatives, into a monophosphate analog and, in particular, 5-FU into 5-FUMP. The expression "mutation" should be understood to mean the addition, deletion and/or substitution of one or more residues at any site of said polypeptide.

The native UPRTase which is referred to in the present invention can be of any origin, in particular of prokaryotic, fungal or yeast origin. By way of illustration, the nucleic acid sequences encoding the UPRTases of *E. coli* (Anderson et al., 1992, Eur. J. Biochem 204, 51-56), of *Lactococcus lactis* (Martinussen and Hammer, 1994, J. Bacteriol. 176, 6457-6463), of *Mycobacterium bovis* (Kim et al., 1997, Biochem Mol. Biol. Int 41, 1117-1124) and of *Bacillus subtilis* (Martinussen et al., 1995, J. Bacteriol. 177, 271-274) can be used in the context of the invention. However, preference is most particularly given to the use of a yeast UPRTase and in particular that encoded by the *S. cerevisiae FUR1* gene, the sequence of which, disclosed in Kern et al. (1990, Gene 88, 149-157), is introduced herein by way of reference. By way of indication, the sequences of the genes and those of the corresponding UPRTases can be found in the literature and in the specialist databanks (SWISSPROT, EMBL, Genbank, Medline, etc.).

Moreover, application PCT/FR99/00904 describes a *FUR1* gene lacking 105 nucleotides in 5' of the coding region, allowing the synthesis of a UPRTase deleted of the first 35 residues in the N-terminal position and starting at the methionine at position 36 in the native protein. The product of expression of the mutant gene, referred to as *FUR1Δ105*, is capable of complementing an *S. cerevisiae fur1* mutant. In addition, the truncated mutant has UPRTase activity greater than that of the native enzyme. Thus, according to a particularly advantageous embodiment, the encoded polypeptide according to the invention is a deletion mutant of a native UPRTase. The deletion is preferably located in the N-terminal region of the UPRTase of origin. It can

be total (concerning all of the residues of said N-terminal region) or partial (concerning one or more residues, which may or may not be continuous, in the primary structure). In general, a polypeptide consists of N-terminal, central and C-terminal portions, each one representing approximately one third of the molecule. For example, since the UPRTase of *S. cerevisiae* has 251 amino acids, its N-terminal portion consists of the first 83 residues starting at the so-called initiating methionine located at the first position of the native form. As for the UPRTase of *E. coli*, its N-terminal portion covers positions 1 to 69.

In addition, patent applications WO 96/16183 and PCT/FR99/00904 describe the use of a fusion protein encoding an enzyme with two domains having the CDase and UPRTase activities, and demonstrate that the transfer of a hybrid gene *codA::upp* or *FCY1::FUR1* or *FCY1::FUR1Δ105* carried by an expression plasmid increases the sensitivity to 5-FC of transfected B16 cells. The protein and nucleic acid sequences described in these two applications are incorporated into the description of the present application. According to this embodiment, the polypeptide is a polypeptide fused, in frame, with at least a second polypeptide. Although the fusion can take place at any site of the first polypeptide, the N- or C-terminal ends are preferred, and in particular the N-terminal end. Fusion of the CDase and UPRTase activities makes it possible to improve the sensitivity of the target cells to 5-FC and to 5-FU.

Those skilled in the art are capable of cloning the CDase or UPRTase sequences using the published data, of carrying out optional mutations, of testing the enzymatic activities of the mutant forms in an acellular or cellular system according to the technology of the art or by following the protocol indicated in application PCT/FR99/00904, and of fusing, in particular in frame, the polypeptides with CDase and

UPRTase activity and, consequently, all or part of the corresponding genes.

Consequently, according to a specific case, the composition of the invention is characterized in that  
5 the nucleic acid sequence (ii) is selected from the nucleic acid sequences of the CodA, upp, FUR1, FCY1 and FUR1Δ105 genes, or by a combination of all or part of said sequences.

The invention relates more particularly to a  
10 said composition characterized in that said polypeptide in (ii) has at least CDase activity and UPRTase activity.

The expression "combination of nucleic acid sequences" is intended to refer both to distinct  
15 sequences which encode at least two distinct polypeptides and to fused sequences which encode fusion polypeptides, it being understood that the production of such polypeptides can be carried out under the control of the same regulation elements (polycistronic cassette) or of independent elements which may be  
20 identical or different, which may be homologous or heterologous with respect to the vector containing them and which may be constitutive or inducible.

According to a particular embodiment, the  
25 composition of the invention comprises at least one nucleic acid sequence (ii) encoding a fusion polypeptide in which a first polypeptide having UPRTase or CDase activity is fused, in frame, with at least a second polypeptide, said second polypeptide having  
30 CDase or UPRTase activity, respectively. More particularly, such a polypeptide is characterized in that the fusion with the second polypeptide is carried out at the N-terminal end of said first polypeptide.

According to one preferred case, said  
35 composition is characterized in that the nucleic acid sequence encoding said fusion polypeptide is a hybrid sequence comprising:

- a first nucleic acid sequence encoding a first polypeptide having UPRTase or CDase activity,

-a second nucleic acid sequence encoding a second polypeptide having CDase or UPRTase activity, respectively.

Such a hybrid nucleic acid sequence encoding  
5 said fusion polypeptide can also contain an IRES-type sequence.

The invention in particular relates to such a composition for which the first nucleic acid sequence is selected from upp, FUR1 and FUR1Δ105, and in that  
10 the second nucleic acid sequence is selected from CodA and FCY1, and vice versa. Most preferably, such a hybrid nucleic acid sequence is chosen from the hybrid sequences described in patent applications WO 96/16183 and PCT/FR99/00904.

15 According to a third variant, the composition according to the present invention is characterized in that said polypeptide having cytotoxic activity (ii) is an anti-angiogenic protein factor. Angiogenesis is the process responsible for the formation of new  
20 capillaries from the already existing vascular network. This complex process is finely regulated in healthy tissues through the balance of the effects of many angiogenic and anti-angiogenic factors. However, in certain pathologies, and in particular in the formation  
25 of a tumor, this process is disrupted: the angiogenic factors override the anti-angiogenic factors, which allows considerable vascularization of the tumors and, consequently, their rapid development and/or the appearance of metastases. This is why, in the context  
30 of the present invention, an anti-angiogenic factor is considered to be a cytotoxic, in particular antitumor, agent. Among the various anti-angiogenic factors known at the current time, mention may be made in particular of angiostatin, endostatin, platelet factor PF4,  
35 thrombospondin-1, PRP (for Proliferin Related Protein), VEGI (for Vascular Endothelial Growth Inhibitor) metalloproteases and urokinase.

The nucleic acid sequences (i) or (ii) can be easily obtained by cloning, by PCR or by chemical

synthesis, according to the conventional techniques in use. They may be native genes or genes derived from the latter by mutation, deletion, substitution and/or addition of one or more nucleotides. Moreover, their sequences are widely described in the literature, which can be consulted by those skilled in the art.

The present invention also relates to a composition as presented above, characterized in that said nucleic acid sequences (i) and (ii) are inserted into a recombinant vector of plasmid or viral origin, and to such a recombinant vector carrying such nucleotide sequences placed under the control of the elements required for their expression in a host cell. The nucleic acid sequences (i) and (ii) can be present in one or more copies on the same vector.

More particularly, the compositions of the invention can comprise said nucleic acid sequences (i) and (ii) inserted into the same recombinant vector or into distinct recombinant vectors.

According to the invention, the expression "recombinant vector" is intended to refer to a vector of plasmid or viral origin, and optionally such a vector associated with one or more substances which improve the efficiency of transfection and/or the stability of said vector and/or the protection of said vector *in vivo* with respect to the immune system of the host organism. These substances are widely documented in the literature accessible to those skilled in the art (see, for example, Felgner et al., 1987, Proc. West. Pharmacol. Soc. 32, 115-121; Hodgson and Solaiman, 1996, Nature Biotechnology 14, 339-342; Remy et al., 1994, Bioconjugate Chemistry 5, 647-654). By way of nonlimiting illustration, they may be polymers, lipids, in particular cationic lipids, liposomes, nuclear or viral proteins, or neutral lipids. These substances can be used alone or in combination. Examples of such compounds are in particular available in patent applications WO 98/08489, WO 98/17693, WO 98/34910, WO 98/37916, WO 98/53853, EP 890362 or

WO 99/05183. A conceivable combination is a recombinant plasmid vector associated with cationic lipids (DOGS, DC-CHOL, spermine-chol, spermidine-chol, etc.) and neutral lipids (DOPE).

5       The choice of the plasmids which can be used in the context of the present invention is vast. They can be cloning and/or expression vectors. In general, they are known to those skilled in the art and many of them are commercially available, but it is also possible to  
10       construct them or modify them using the techniques of genetic manipulation. By way of example, mention may be made of the plasmids derived from pBR322 (Gibco BRL), pUC (Gibco BRL), pBluescript (Stratagene), pREP4, pCEP4 (Invitrogen) or p Poly (Lathe et al., 1987, Gene 57,  
15       193-201). Preferably, a plasmid used in the context of the present invention contains an origin of replication which ensures the initiation of the replication in a producer cell and/or a host cell (for example, the ColE1 origin will be selected for a plasmid intended to  
20       be produced in *E. coli* and the oriP/EBNA1 system will be selected if self-replication of the plasmid in a mammalian host cell is desired, Lupton and Levine, 1985, Mol. Cell. Biol. 5, 2533-2542; Yates et al., Nature 313, 812-815). It can also comprise a selection  
25       gene making it possible to select or identify the cells transfected (complementation of an auxotrophy mutation, gene encoding resistance to an antibiotic, etc.). Of course, it can comprise additional elements which improve its persistence and/or its stability in a given  
30       cell (cer sequence which promotes the monomeric persistence of a plasmid (Summers and Sherrat, 1984, Cell 36, 1097-1103, sequences for integration into the cellular genome).

      With regard to a viral vector, it is possible  
35       to envisage a vector deriving from a poxvirus (vaccinia virus, in particular MVA, canarypox, etc.), from an adenovirus, from a retrovirus, from a herpesvirus, from an alphavirus, from a foamyvirus or from an adeno-associated virus. Use will preferably be made of a



nonreplicating and nonintegrating vector. In this respect, the adenoviral vectors are most particularly suitable for the use of the present invention. However, it should be noted herein that, in the context of the use of the present invention, the nature of the vector is relatively unimportant.

Retroviruses have the property of infecting and of integrating mainly in dividing cells and, in this respect, are particularly suitable for the cancer application. A recombinant retrovirus according to the invention generally comprises the LTR sequences, an encapsidation region and the nucleotide sequence according to the invention placed under the control of the retroviral LTR or of an internal promoter such as those described below. It can derive from a retrovirus of any origin (murine, primate, feline, human, etc.), and in particular from the MoMuLV (Moloney murine leukemia virus), MSV (Murine sarcoma virus) or Friend murine retrovirus (Fb29). It is propagated in an encapsidation line capable of providing, *in trans*, the gag, pol and/or env viral polypeptides required for the constitution of a viral particle. Such lines are described in the literature (PA317, Psi CRIP GP + Am-12, etc.). The retroviral vector according to the invention can comprise modifications in particular in the LTRs (replacement of the promoter region with a eukaryotic promoter) or in the encapsidation region (replacement with a heterologous encapsidation region, for example of VL30 type) (see French applications 94/08300 and 97/05203).

Use may also be made of an adenoviral vector defective for replication, i.e. lacking all or part of at least one region essential for replication, selected from the E1, E2, E4 and [lacuna] regions. A deletion of the E1 region is preferred. However, it can be combined with other modification(s)/deletion(s) affecting in particular all or part of the E2, E4 and/or L1-L5 regions, insofar as the defective essential functions are complemented, *in trans*, by means of a

complementation line and/or of an auxiliary virus, in order to ensure the production of the viral particles of interest. In this respect, use can be made of the second generation vectors of the state of the art (see, 5 for example, international applications WO 94/28152 and WO 97/04119). By way of illustration, the deletion of the majority of the E1 region and of the E4 transcription unit is most particularly advantageous. With the aim of increasing the cloning capacities, the 10 adenoviral vector can also lack all or part of the nonessential E3 region. According to another alternative, it is possible to use a minimum adenoviral vector retaining the sequences essential for the encapsidation, i.e. the 5' and 3' ITRs (Inverted 15 Terminal Repeats) and the encapsidation region. Moreover, the origin of the adenoviral vector according to the invention can be varied from the point of view of both the species and the serotype. It can derive from the genome of an adenovirus of human or animal 20 (canine, avian, bovine, murine, ovine, porcine, simian, etc.) origin or of a hybrid comprising fragments of adenoviral genome of at least two different origins. Mention may be made more particularly of the CAV-1 or CAV-2 adenovirus of canine origin, the DAV adenovirus 25 of avian origin or the type 3 Bad adenovirus of bovine origin (Zakharchuk et al., Arch. Virol. 1993, 128: 171-176; Spibey and Cavanagh, J. Gen. Virol., 1989, 70: 165-172; Jouvenne et al., Gene, 1987, 60: 21-28; Mittal et al., J. Gen. Virol., 1995, 76: 93-102). However, 30 preference will be given to an adenoviral vector of human origin, preferably deriving from a serotype C adenovirus, in particular a type 2 or 5 adenovirus. An adenoviral vector according to the present invention can be generated in vitro in Escherichia coli (E. coli) 35 by ligation or homologous recombination (see, for example, international application WO 96/17070) or by recombination in a complementation line. The various adenoviral vectors and the techniques for preparing them are known (see, for example, Graham and Prevec,

1991, in *Methods in Molecular Biology*, vol 7, p 109-128; Ed: E.J. Murey, The Human Press Inc).

The elements required for expression consist of all of the elements allowing the transcription of the nucleotide sequence into RNA and the translation of the mRNA into a polypeptide, in particular the promoter sequences and/or regulation sequences which are effective in said cell and, optionally, the sequences required for the excretion or expression of said polypeptide at the surface of the target cells. These elements can be regulatable or constitutive. Of course, the promoter is suitable for the vector selected and for the host cell. By way of examples, mention may be made of the eukaryotic promoters of the PGK (Phosphoglycerate Kinase), MT (metallothionein; McIvor et al., 1987, *Mol. Cell. Biol.* 7, 838-848),  $\alpha$ -1 antitrypsin and CFTR genes, the promoters of the gene encoding muscle creatine kinase, actin, lung surfactant, immunoglobulin,  $\beta$ -actin (Tabin et al., 1982, *Mol. Cell. Biol.* 2, 426-436) and SR $\alpha$  (Takebe et al., 1988, *Mol. Cell. Biol.* 8, 466-472), the SV40 virus (Simian Virus) early promoter, the RSV (Rous Sarcoma Virus) LTR, the MPSV promoter, the HSV-1 TK promoter, the CMV virus (cytomegalovirus) early promoter, the vaccinia virus promoters p7.5K, pH5R, Pk1L, p28 and p11 and the E1A and MLP adenoviral promoters, or a combination of said promoters. It can also be a promoter which stimulates expression in a cancerous or tumor cell. Mention may be made, in particular, of the promoters of the MUC-1 gene overexpressed in breast cancers and prostate cancers (Chen et al., 1995, *J. Clin. Invest.* 96, 2775-2782), CEA (for carcinoma embryonic antigen) gene overexpressed in colon cancers (Schrewe et al., 1990, *Mol. Cell. Biol.* 10, 2738-2748), tyrosinase gene overexpressed in melanomas (Vile et al., 1993, *Cancer Res.* 53, 3860-3864), ERB-2 gene overexpressed in breast cancers and cancers of the pancreas (Harris et al., 1994, *Gene Therapy* 1, 170-175) and  $\alpha$ -fetoprotein gene overexpressed in liver cancers

(Kanai et al., 1997, Cancer Res. 57, 461-465). The Cytomegalovirus (CMV) early promoter, or that of the RSV, is most particularly preferred. It is also possible to use a promoter region which is tissue  
5 specific, in particular when the tumor to be treated is derived from a specific cell type, or which can be activated under defined conditions. The literature provides a large amount of information relating to such promoter sequences.

10 The required elements can also include additional elements which improve the expression of the nucleotide sequence according to the invention or its persistence in the host cell. Mention may be made, in particular, of intronic sequences (WO 94/29471),  
15 secretion signal sequences, nuclear localization sequences, IRES-type internal translation reinitiation sites and poly A transcription termination sequences.

According to a preferred embodiment, the invention relates more particularly to a recombinant  
20 vector, in particular a viral vector, and more specifically to an adenoviral vector defective for replication, comprising:

- (i) a nucleic acid sequence encoding all or part of an MIP chemokine,
- 25 (ii) at least one nucleic acid sequence encoding all or part of a polypeptide having at least cytotoxic activity,

said nucleic acid sequences being placed under the control of the elements required for their expression  
30 in a host cell and being defined as indicated above.

A subject of the present invention is also a viral particle, in particular an adenoviral particle, comprising a recombinant viral vector according to the invention. Such a viral particle can be generated from  
35 a viral vector according to any conventional technique in the field of the art. Its propagation is carried out in particular in a complementation cell suited to the deficiencies of said vector. With regard to an adenoviral vector, use will, for example, be made of a

complementation line as described in application WO 94/28152, of the 293 line established from human embryonic kidney cells which effectively complements the E1 function (Graham et al., 1977, J. Gen. Virol. 36, 59-72), the A549-E1 line (Imler et al., 1996, Gene Therapy 3, 75-84) or a line allowing double complementation (Yeh et al., 1996, J. Virol. 70, 559-565; Krougliak and Graham, 1995, Human Gene Therapy 6, 1575-1586; Wang et al., 1995 Gene Therapy 2, 775-783; international application WO 97/04119). It is also possible to use auxiliary viruses to complement, at least in part, the defective functions. The expression "complementation cell" is intended to mean a cell capable of providing, *in trans*, the early and/or late factors required for the encapsidation of the viral genome in a viral capsid, in order to generate a viral particle containing the recombinant vector. Said cell may, by itself, not complement all the defective functions of the vector and, in this case, may be transfected/transduced with an auxiliary virus/vector which provides the complementary functions.

The invention also relates to a method for preparing a viral particle, according to which:

- (i) a recombinant vector according to the invention is introduced into a cell, in particular a complementation cell capable of complementing, *in trans*, said vector, so as to obtain a said transfected cell,
- (ii) said transfected cell is cultured under suitable conditions in order to allow the production of said viral particle, and
- (iii) said viral particle is recovered from the cell culture.

Of course, the viral particle can be recovered from the culture supernatant, but also from the cells. One of the methods commonly used consists in lysing the cells with consecutive cycles of freezing/thawing, in

order to harvest the virions in the lysis supernatant. These virions can be amplified and purified according to the techniques of the art (chromatographic method, ultracentrifugation in particular through a cesium chloride gradient, etc.).

The invention also relates to a eukaryotic host cell comprising the DNA fragments present in the composition according to the invention. Said host cell is advantageously a mammalian cell and, preferably, a human cell. It will preferably be a 293, LCA4 or PERC6 cell. Such a cell is in particular useful for producing the viral particles at high titer without generating replication-competent particles. The invention also relates to a host cell comprising a nucleotide sequence or a recombinant vector according to the invention, or infected with a viral particle according to the invention. For the purposes of the present invention, a host cell consists of any cell which can be transfected with a recombinant vector or infected with a viral particle, as defined above. A mammalian, and in particular human, cell is most particularly suitable. It can comprise said vector in a form integrated in the genome or not integrated in the genome (episome). It can be a primary or tumor cell of any origin, in particular hematopoietic (totipotent stem cell, leukocyte, lymphocyte, monocyte or macrophage, etc.), muscle (satellite cell, myocyte, myoblast, smooth muscle cell, etc.), cardiac, pulmonary, tracheal, hepatic, epithelial or fibroblast origin.

The invention also relates to a composition intended for the implementation of an antitumor or antiviral treatment, or any applications requiring cell death, in mammals, comprising:

- (i) all or part of the MIP polypeptide,
  - (ii) all or part of a polypeptide having at least cytotoxic activity,
- said polypeptides being defined as indicated above.

Another subject according to the invention consists of a formulation intended for the implementation of a cytotoxic, in particular antitumor or antiviral, treatment in mammals, characterized in that it comprises a composition (based on nucleic acids or on polypeptides as described above), an adenoviral vector or a viral particle according to the invention, and a support which is acceptable from a pharmaceutical point of view. Such a support is preferably isotonic, hypotonic or weakly hypertonic and has a relatively low ionic strength, such as for example a sucrose solution. Moreover, such a support can contain any solvent, or aqueous or partially aqueous liquid such as nonpyrogenic sterile water. The pH of the formulation is also adjusted and buffered in order to satisfy the requirements of use *in vivo*. The formulation can also include a diluent, an adjuvant or an excipient, which is acceptable from a pharmaceutical point of view, as well as solubilization, stabilization, preservation agents. For injectable administration, a formulation in aqueous, nonaqueous or isotonic solution is preferred. It can be in a single dose or in multidoses in liquid form or dry form (powder, lyophilizate, etc.) capable of being reconstituted extemporaneously with a suitable diluent.

According to a particular embodiment of the invention, said formulation also comprises amounts which are acceptable from a pharmaceutical point of view of prodrug capable of being transformed into a cytotoxic molecule by a polypeptide having at least cytotoxic activity.

Such a prodrug will in particular be selected from the group consisting of acyclovir or of gancyclovir (GCV), cyclophosphamide, 6-methylpurine deoxyribonucleoside, 6-thioxanthine, cytosine or one of its derivatives, or uracil or one of its derivatives. Entirely preferably, said prodrug is 5-fluorocytosine (5FC) or 5-fluorouracil (5-FU).

Moreover, in particular in the context of formulations containing a composition according to the second variant mentioned above, it should be noted that said formulation can also comprise one or more substances which potentiate the cytotoxic effect of 5-FU. Mention may be made, in particular, of drugs which inhibit the enzymes of the *de novo* biosynthesis pathway of pyrimidines (for example, those mentioned below), drugs such as Leucovorin (Waxman et al., 1982, Eur. J. Cancer Clin. Oncol. 18, 685-692) which, in the presence of the product of 5-FU metabolism (5-FdUMP), increases the inhibition of thymidylate synthase, which causes a decrease in the pool of dTMP required for replication, and finally, drugs such as methotrexate (Cadman et al., 1979, Science 250, 1135-1137) which, by inhibiting dihydrofolate reductase and increasing the pool of incorporation of PRPP (phosphoribosyl pyrophosphate) causes an increase in 5-FU in the cellular RNA.

A formulation according to the invention is more particularly intended for the preventive or curative treatment of diseases by gene therapy and is directed more particularly at proliferative diseases (cancers, tumors, restenosis, etc.) and at diseases of infectious, in particular viral, origin for which it is necessary to limit the proliferation of the infected cells (induced by the hepatitis B or C viruses, HIV, herpesvirus, retroviruses, etc.).

A formulation according to the invention can be manufactured conventionally with a view to local, parenteral or digestive administration. Many routes of administration can be envisaged. Mention may be made, for example, of the intragastric, subcutaneous, intracardiac, intramuscular, intravenous, intraperitoneal, intratumoral, intranasal, intrapulmonary or intratracheal route. For the three latter embodiments, administration by aerosol or instillation is advantageous. The administration can take place in a single dose or in doses repeated one or more times



after a certain time delay. The suitable route of administration and dose of virus vary as a function of various parameters, for example of the individual, of the disease to be treated or of the gene(s) of interest to be transferred. The preparations based on viral particles according to the invention can be formulated in the form of doses of between  $10^4$  and  $10^{14}$  pfu (plaque forming units), advantageously  $10^5$  and  $10^{13}$  pfu, and preferably  $10^6$  and  $10^{12}$  pfu. With regard to the recombinant vector according to the invention, doses comprising from 0.01 to 100 mg of DNA, preferably 0.05 to 10 mg, and most preferably 0.05 to 5 mg can be envisaged. A composition based on polypeptides preferably comprises from 0.05 to 10 g, and most preferably from 0.5 to 5 g of said polypeptide. Of course, the doses can be adjusted by the clinician.

The present invention also relates to the therapeutic or prophylactic use of a composition, of a recombinant vector or of a viral particle according to the invention, for preparing a medicinal product intended for the treatment of the human or animal body by gene therapy, in particular for preparing a cytotoxic, in particular antitumor or antiviral, medicinal product intended to inhibit the growth or cause the rejection of a tumor, or the death of an infected cell. According to a first possibility, the medicinal product can be administered directly *in vivo* (for example, by intravenous injection, into an accessible tumor or at its periphery, into the lungs by aerosol, into the vascular system by means of a suitable probe, etc.). It is also possible to adopt the *ex vivo* approach, which consists in removing cells from the patient (bone marrow stem cells, peripheral blood lymphocytes, muscle cells, etc.), transfecting or infecting them *in vitro* according to the techniques of the art and readministering them to the patient. A preferred use consists in treating or preventing cancers, tumors and diseases resulting from an undesired cell proliferation. Among the applications

which can be envisaged, mention may be made of breast cancers, cancers of the uterus (in particular those induced by papilloma viruses), cancers of the prostate, lung cancers, bladder cancers, liver cancers, colon  
5 cancers, cancers of the pancreas, stomach cancers and cancers of the esophagus, of the larynx, of the central nervous system and of the blood (lymphomas, leukemia, etc.). It is also useful in the context of cardiovascular diseases, for example for inhibiting or  
10 delaying the proliferation of the smooth muscle cells of the vascular wall (restenosis). Finally, with regard to infectious diseases, use in AIDS can be envisaged.

It is, moreover, conceivable, where appropriate and without straying from the context of the present  
15 invention, to carry out simultaneous or successive administrations, via different routes, of the various components included in the pharmaceutical formulation or composition according to the invention.

The invention also extends to a method for  
20 treating diseases by gene therapy, characterized in that a nucleotide sequence, a recombinant vector, a viral particle or a host cell according to the invention is administered to an organism or to a host cell needing such a treatment.

When the treatment method uses a nucleotide  
25 sequence, a recombinant vector or a viral particle allowing the expression of a polypeptide according to the invention having UPRTase activity, it may be advantageous to also administer a second nucleotide  
30 sequence encoding a second polypeptide having CDase activity, said second nucleotide sequence being carried by said recombinant vector or viral particle or by a vector or an independent viral particle. In the latter case, the administration of the UPRTase and CDase  
35 sequences can be simultaneous or consecutive, the order of administration having no importance.

According to an advantageous embodiment, the therapeutic use or the treatment method also comprise an additional step according to which amounts which are

acceptable from a pharmaceutical point of view of a prodrug, advantageously of a cytosine analog, and in particular of 5-FC, are administered to the host cell or organism. By way of illustration, a dose of 50 to 500 mg/kg/day can be used, with a preference for 200 mg/kg/day. In the context of the present invention, the prodrug is administered according to standard practices, this being prior to, concomitant with or after the administration of the therapeutic agent according to the invention. The oral route is preferred. It is possible to administer a single dose of prodrug or doses repeated for a sufficiently long time so as to allow the production of the toxic metabolite in the host cell or organism.

According to an advantageous embodiment of the invention, the therapeutic use or the treatment method is combined with a second treatment of the patient by surgery (in particular by partial or total ablation of the tumor), by radiotherapy or chemotherapy. In this particular case, the treatment according to the invention is used prior to or concomitant with, or is carried out subsequent to, said second treatment. Preferably, this treatment will be used subsequent to said second treatment.

The aim of the examples which follow is to illustrate the various subjects of the present invention and, consequently, they are in no way limiting in nature.

Figure 1 represents the evolution of the tumor volume in B6D2 mice implanted with B16F0 tumor cells.

Figure 2 represents the survival rate of these same mice. Groups of 15 mice are treated using compositions comprising adenoviruses expressing the following genes: huMIP $\alpha$ , huIL2, huMIP1 $\alpha$  + huIL2, empty Ad.

Figure 3 represents the evolution of the tumor volume in B6D2 mice implanted with RENCA tumor cells.

Figure 4 represents the survival rate of these same mice. Groups of 15 mice are treated using

compositions comprising adenoviruses expressing the following genes: huMIP $\beta$ , huIL2, huMIP $\beta$  + huIL2, empty Ad.

Figure 5 represents the evolution of the tumor volume in B6D2 mice implanted with p815 tumor cells.

Figure 6 represents the survival rate of these same mice. Groups of 15 mice are treated using compositions comprising adenoviruses expressing the following genes: Tris buffer, huMIP $\alpha$  + huIL2, huMIP $\alpha$  + muIFN $\gamma$ , huIL2 + muIFN $\gamma$ .

Figure 7 represents the evolution of the tumor volume in B6D2 mice implanted with RENCA tumor cells. These mice are treated using compositions comprising adenoviruses expressing the human MIP1 $\beta$  gene (huMIP1 $\beta$ ) in combination with adenoviruses expressing the murine IL12 gene (muIL12), or adenoviruses expressing the muIL12 gene, or adenoviruses containing no transgene (empty Ad).

#### EXAMPLES:

The constructs described below are prepared according to the general techniques of genetic engineering and of molecular cloning detailed in Maniatis et al., (1989, Laboratory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, NY) or according to the manufacturer's recommendations when a commercial kit is used. The homologous recombination steps are preferably carried out in the *E. coli* strain BJ 5183 (Hanahan, 1983, *J. Mol. Biol.* 166, 557-580). With regard to the repair of the restriction sites, the technique used consists in filling the protruding 5' ends using the large fragment of *E. coli* DNA polymerase I (Klenow). Moreover, the fragments of adenoviral genome used in the various constructs described hereinafter are precisely indicated according to their position in the nucleotide sequence of the Ad5 genome as disclosed in the Genbank databank under the reference M73260.

With regard to the cell biology, the cells are transfected or transduced and cultured according to the standard techniques well known to those skilled in the art.

5           *Tumor models:*

Three tumor cell models were chosen in order to evaluate the activity of the composition of the invention: P815 (H-2d mastocytoma, described in Dunn et al, 1957, J.Natl. Cancer Inst., 18, 587-590), B16FO  
10 (H-2b melanoma, described in Wu et al, 1996, Cancer Res., 56, 21-26) and RENCA (H-2d renal carcinoma, described in Murphy et al, 1973, J.Natl.Cancer Inst., 50(4), 1023-1025). The cells (3 for each tumor model) are implanted on D-7/D-11, subcutaneously in the right  
15 flank of 6- to 8-week-old B6D2 mice.

*Administration of the cytotoxic compositions of the invention:*

A 100 µl volume of adenoviral vectors ( $5 \times 10^8$  infectious units) is injected directly into the tumors  
20 when their volume is in the region of 4 to 10 mm<sup>3</sup> (D0). This injection is repeated under the same conditions on D1 and D2.

The effectiveness of the composition of the invention administered is controlled by measuring the  
25 size of the tumors and by measuring the survival time of the mice treated with, where appropriate, a control of the immunological status of the animal by ELISPOT, CTL assay, etc. The animals can also then be subjected to a contralateral challenge during which a lethal dose  
30 of tumor cells is administered to the pretreated animal.

**EXAMPLE 1:**

*Construction of pTG13010 (huMIP1α)*

35           The human MIPα cDNA (accession number at GenBank: X03754; sequence incorporated into the application by way of reference) was assembled with synthetic oligonucleotides following the sequence described by Obaru, K. et al., 1986, J. Biochem. 99

(3), 885-894. This cDNA was introduced into a vector derived from pBluescript, in order to give the vector pTG13006.

- The NotI-Asp718 fragment of pTG13006 containing the MIP1 $\alpha$  gene is isolated and introduced into the vector pTG8347 cleaved with these same enzymes, in order to give the transfer vector pTG13008. By way of indication, pTG8347 is a p polyII vector (Lathe et al., 1987, Gene 57, 193-201) into which the Ad5 sequences 1 to 458, the RSV promoter, the splicing sequences of intron 2 of rabbit beta-globin 1, the polyadenylation sequences of rabbit beta-globin 1 and the Ad5 sequences 3328-5788 are inserted. Such a construct is within the scope of those skilled in the art, in particular on the basis of French application 97/06757. The adenoviral vector pTG13010 is reconstituted by recombination, in the *E. coli* strain BJ 5183, between the PacI-BstEII fragment of pTG13008 and the vector pTG6624 (described in French application 97/06757) linearized with ClaI.
- By way of indication, pTG6624 corresponds to the p polyII plasmid carrying the Ad5 genome deleted of the E1 (nt 459 to 3327) and E3 (nt 28592 to 30470) regions, the cassette for expression of MIP being inserted in place of E1.
- The final construct pTG13010 contains the Ad5 genome deleted of most of the E1 (nt 459 to 3328) and E3 (nt 28249 to 30758) regions and, in place of E1, a cassette for the expression of the MIP1 $\alpha$  gene placed under the control of the RSV promoter and of the splicing sequences of intron 2 of rabbit beta-globin 1. The adenoviral particles are generated by transfection into an E1 function complementation line, for example the 293 line (ATCC CRL1573), according to the techniques of the art (Graham and Prevec, 1991, Methods in Molecular Biology Vol 7, Gene Transfer and Expression Protocols; Ed E.J. Murray, The Human Press Inc., Clinton, NJ).

Construction of pTG13023 (huMIP1 $\beta$ /variant Act2).

The human MIP1 $\beta$  cDNA (GenBank accession number: J04130; sequence incorporated into the application by way of reference) was assembled with synthetic oligonucleotides following the sequence described by  
5 Lipes, M.A. et al. 1988, Proc. Natl. Acad. Sci. U.S.A. 85 (24), 9704-9708. This cDNA was introduced into a vector derived from M13TG130 (Kieny et al. 1983, Gene, 26, 91-99), in order to give the vector M13TG13013.

The NotI-Asp718 fragment of M13TG13013  
10 containing the MIP1 $\beta$  gene is isolated and introduced into the vector pTG8347 cleaved with these same enzymes, in order to give the transfer vector pTG13015. Such a construct is within the scope of those skilled in the art, in particular on the basis of French  
15 application 97/06757. The adenoviral vector pTG13023 is reconstituted by recombination, in the *E. coli* strain BJ 5183, between the PacI-BstEII fragment of pTG13015 and the vector pTG6624 (described in French application 97/06757) linearized with ClaI.

20 The final construct pTG13023 contains the Ad5 genome deleted of most of the E1 (nt 459 to 3328) and E3 (nt 28249 to 30758) regions and, in place of E1, a cassette for the expression of the MIP1 $\beta$  gene placed under the control of the RSV promoter and of the  
25 splicing sequences of intron 2 of rabbit  $\beta$  globin 1. The adenoviral particles are generated by transfection into an E1 function complementation line, for example the 293 line (ATCC CRL1573), according to the techniques of the art (Graham and Prevec, 1991, Methods  
30 in Molecular Biology Vol 7, Gene Transfer and Expression Protocols; Ed E.J. Murray, The Human Press Inc., Clinton, NJ).

#### **EXAMPLE 2:**

35 Experiments *in vivo*.

In order to evaluate the capacity of the compositions of the invention to inhibit the growth of tumors *in vivo*,  $3 \times 10^5$  B16F0, RENCA or P815 cells are injected on (D = -10/-7) into B6D2 immunocompetent

mice. As soon as the tumors become palpable ( $D = 0$ ) various compositions (see the legend of Figures 1 to 6) are injected three times ( $D_0$ ,  $D_1$ ,  $D_2$ ), intratumorally, at a dose of  $5 \times 10^8$  infectious units.

5       The results obtained demonstrate an increase in the survival rates (Figures 1, 3 and 5) associated with a decrease in the tumor volumes (Figures 2, 4 and 6) in the mice treated with the compositions comprising an adenovirus expressing MIP1 $\alpha$  or MIP1 $\beta$  associated with  
10 IL2 or IFN $\gamma$ . These results clearly confirm the advantage of the compositions of the invention in the implementation of an antitumor treatment.

15       The mice thus treated are then subjected to a contralateral challenge consisting of administration, under the conditions described previously, of a lethal dose ( $3 \times 10^5$  cells) of tumor cells on D80/D100. It was thus noted that the results described above are also accompanied by an immune condition of the mouse such that no tumor is capable of developing after this  
20 challenge step.

### EXAMPLE 3:

#### Experiments *in vivo*

25       In order to evaluate the capacity of the compositions of the invention to inhibit the growth of tumors *in vivo*,  $4 \times 10^5$  RENCA cells are injected on ( $D = 0$ ) into B6D2 immunocompetent mice. As soon as the tumors become palpable ( $D = 6$ ), various compositions containing either  $2 \times 10^8$  infectious units (iu) of an  
30 adenovirus expressing the human MIP1 $\beta$  gene (huMIP1 $\beta$ ) in combination with  $2 \times 10^8$  iu of an adenovirus expressing the murine IL12 gene (muIL12), or  $2 \times 10^8$  iu of an adenovirus expressing the muIL12 gene in combination with  $2 \times 10^8$  iu of an adenovirus containing no transgene  
35 (empty Ad), or  $4 \times 10^8$  iu of an empty Ad, are injected three times ( $D_6$ ,  $D_7$ ,  $D_8$ ), intratumorally. For all these compositions, the adenoviruses are in a solution containing 100 mM of Tris and 10 mM of MgCl $_2$ . It was, moreover, verified that the mice treated, under the



same conditions, with a composition comprising only the adenoviruses expressing the MIP1 $\beta$  gene exhibited tumors with a volume identical to or even greater than those observed in mice treated with a composition comprising empty Ads.

The results obtained according to this example (Figure 7) show a decrease in the tumor volumes in the mice treated with the compositions of the invention comprising an adenovirus expressing MIP1 $\beta$  combined with an adenovirus expressing IL12, most particularly in comparison with the results observed during the treatment of mice with muIL12 alone. These results clearly confirm the advantage of the compositions of the invention in the implementation of an antitumor treatment.

## CLAIMS

1. Composition intended for the implementation of a cytotoxic treatment in mammals, comprising:

- (i) a nucleic acid sequence encoding all or  
5 part of an MIP chemokine,  
(ii) at least one nucleic acid sequence encoding all or part of a polypeptide having at least cytotoxic activity,

said nucleic acid sequences being placed under  
10 the control of the elements required for their expression in a host cell of said mammal.

2. Composition according to Claim 1, characterized in that said MIP chemokine is the MIP1 chemokine, and more particularly selected from the group consisting of  
15 the MIP1 $\alpha$  and MIP1 $\beta$  chemokines.

3. Composition according to either of Claims 1 and 2, characterized in that said polypeptide having cytotoxic activity is chosen from cytokines, proteins encoded by suicide genes and anti-angiogenic protein  
20 factors.

4. Composition according to Claim 3, characterized in that said polypeptide having cytotoxic activity is a cytokine chosen from interferons  $\alpha$ ,  $\beta$  and  $\gamma$ , interleukins, tumor necrosis factors and colony  
25 stimulating factors.

5. Composition according to Claim 4, characterized in that said polypeptide having cytotoxic activity is interleukin-2 (IL-2).

6. Composition according to Claim 4, characterized  
30 in that said polypeptide having cytotoxic activity is interferon gamma (IFN- $\gamma$ ).

7. Composition according to one of Claims 1 to 6, characterized in that it comprises in (ii) at least two nucleic acid sequences encoding all or part of  
35 interleukin-2 (IL-2) and all or part of interferon gamma (IFN- $\gamma$ ).

8. Composition according to Claim 4, characterized in that said polypeptide has at least a cytotoxic

activity selected from thymidine kinase activity, purine nucleoside phosphorylase activity, guanine phosphoribosyl transferase activity and cytosine deaminase activity.

5 9. Composition according to Claim 8, characterized in that said polypeptide has at least CDase activity and UPRTase activity.

10. Composition according to Claim 4, characterized in that said polypeptide having cytotoxic activity is  
10 an anti-angiogenic protein factor chosen from angiostatin, endostatin, platelet factor PF4, thrombospondin-1, PRP, VEGF, metalloproteases and urokinase.

11. Composition according to Claim 1, characterized  
15 in that said nucleic acid sequences (i) and (ii) are inserted into a recombinant vector of plasmid or viral origin.

12. Composition according to Claim 11,  
characterized in that said nucleic acid sequences (i)  
20 and (ii) are inserted into the same recombinant vector.

13. Composition according to Claim 11,  
characterized in that said nucleic acid sequences (i)  
and (ii) are inserted into distinct recombinant  
vectors.

25 14. Vector comprising:

(i) a nucleic acid sequence encoding all or  
part of an MIP chemokine,

(ii) at least one nucleic acid sequence  
encoding all or part of a polypeptide having at least  
30 cytotoxic activity,

said nucleic acid sequences being placed under the  
control of the elements required for their expression  
in a host cell.

15. Vector according to Claim 14, characterized in  
35 that it is a viral vector.

16. Viral particle comprising a vector according to  
Claim 15.

17. Method for preparing a viral particle according  
to Claim 16, according to which:

(i) a viral vector according to claim 15 is introduced into a cell capable of producing said vector, so as to obtain a transfected cell,

(ii) said transfected cell is cultured under  
5 suitable conditions in order to allow the production of said viral particle, and

(iii) said viral particle is recovered from the cell culture.

18. Composition intended for the implementation of  
10 a cytotoxic treatment in mammals, comprising:

(i) all or part of an MIP polypeptide,

(ii) all or part of a polypeptide having at least cytotoxic activity,

according to which said polypeptides (i) and  
15 (ii) are as defined in Claims 1 to 10.

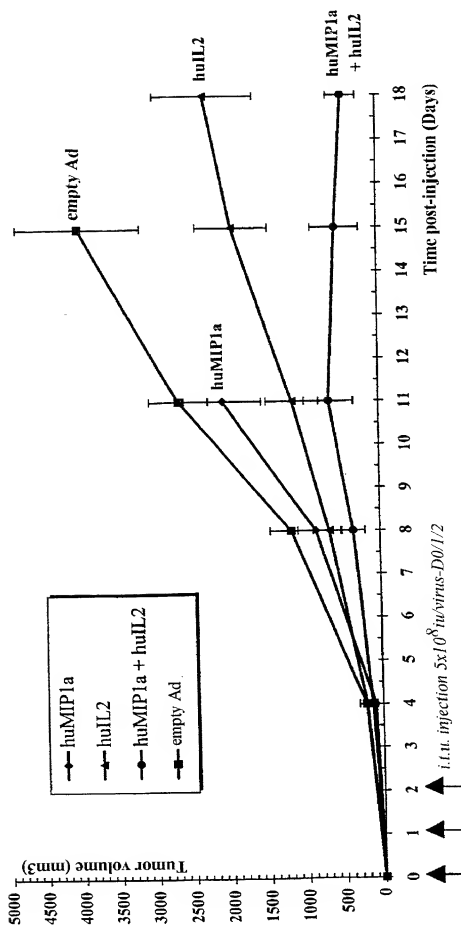
19. Formulation intended for the implementation of a cytotoxic treatment in mammals, characterized in that it comprises a composition according to one of Claims 1 to 13, a vector according to Claims 14 or 15, a viral  
20 particle according to Claim 16 or a composition according to according to Claim 18, and a support which is acceptable from a pharmaceutical point of view.

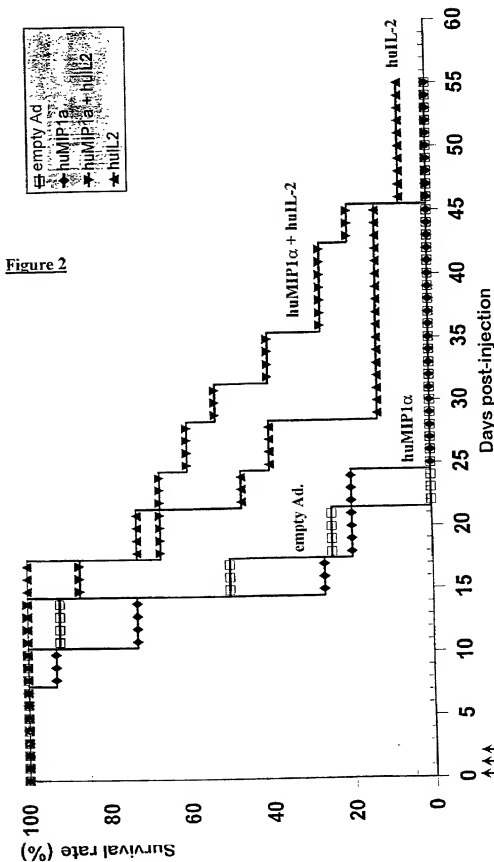
20. Formulation according to Claim 19, characterized in that it comprises amounts which are  
25 acceptable from a pharmaceutical point of view of a prodrug capable of being transformed into a cytotoxic molecule by a polypeptide having at least cytotoxic activity.

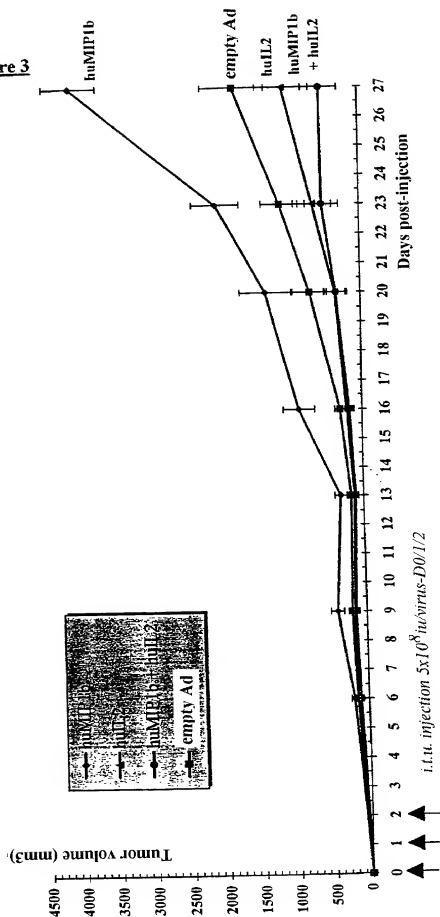
21. Formulation according to Claim 20,  
30 characterized in that said prodrug is selected from 5-fluorouracil (5-FU) and 5-fluorocytosine (5-FC).

22. Use of a composition according to Claims 1 to 13, of a vector according to Claims 14 to 15, of a viral particle according to Claim 16 or of a  
35 composition according to according to Claim 18, for preparing a cytotoxic medicinal product.

Figure 1





**Figure 3**

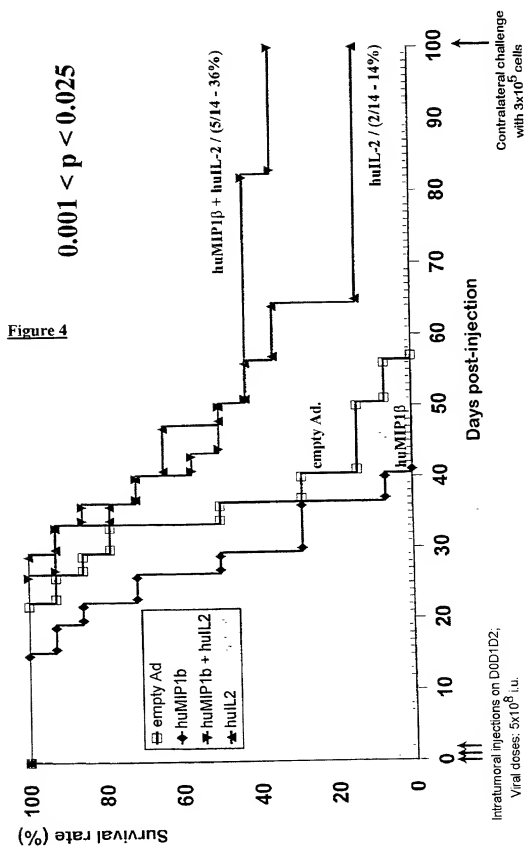
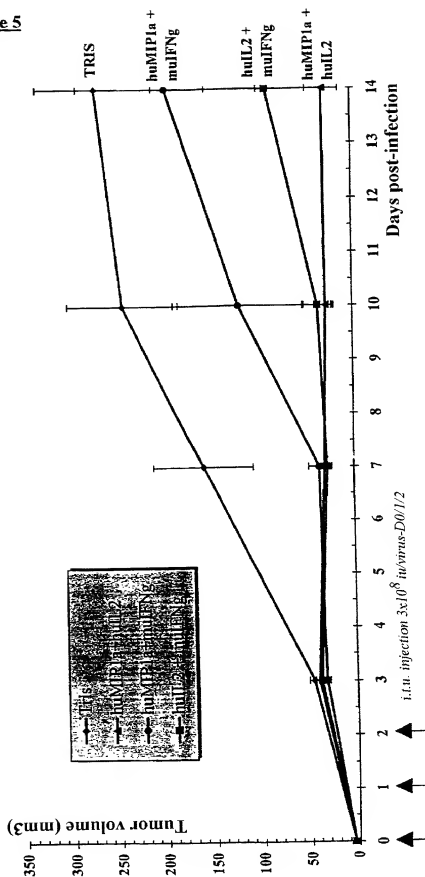




Figure 5



COMBINED DECLARATION AND POWER OF ATTORNEY  
FOR UTILITY PATENT APPLICATION

Attorney's Docket No.

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I BELIEVE I AM THE ORIGINAL, FIRST AND SOLE INVENTOR (if only one name is listed below) OR AN ORIGINAL, FIRST AND JOINT INVENTOR (if more than one name is listed below) OF THE SUBJECT MATTER WHICH IS CLAIMED AND FOR WHICH A PATENT IS SOUGHT ON THE INVENTION ENTITLED:

COMPOSITION INTENDED FOR THE IMPLEMENTATION OF A CYTOTOXIC, IN PARTICULAR

ANTITUMOR OR ANTIVIRAL, TREATMENT IN MAMMALS.

the specification of which

(check one) ☐ is attached hereto;

☒ was filed on June 7th, 2000 as

International Application No. PCT/FR/00/01559

and was amended on \_\_\_\_\_;  
(if applicable)

I HAVE REVIEWED AND UNDERSTAND THE CONTENTS OF THE ABOVE-IDENTIFIED SPECIFICATION, INCLUDING THE CLAIMS, AS AMENDED BY ANY AMENDMENT REFERRED TO ABOVE;

I ACKNOWLEDGE THE DUTY TO DISCLOSE TO THE OFFICE ALL INFORMATION KNOWN TO ME TO BE MATERIAL TO PATENTABILITY AS DEFINED IN TITLE 37, CODE OF FEDERAL REGULATIONS, Sec. 1.56 (as amended effective March 16, 1992);

I do not know and do not believe the said invention was ever known or used in the United States of America before my or our invention thereof, or patented or described in any printed publication in any country before my or our invention thereof or more than one year prior to said application; that said invention was not in public use or on sale in the United States of America more than one year prior to said application; that said invention has not been patented or made the subject of an inventor's certificate issued before the date of said application in any country foreign to the United States of America on any application filed by me or my legal representatives or assigns more than twelve months prior to said application;

I hereby claim foreign priority benefits under Title 35, United States Code Sec. 119 and/or Sec. 365 of any foreign application(s) for patent or inventor's certificate as indicated below and have also identified below any foreign application for patent or inventor's certificate on this invention having a filing date before that of the application(s) on which priority is claimed:

## COMBINED DECLARATION AND POWER OF ATTORNEY

Attorney's Docket No.

COUNTRY/INTERNATIONAL

APPLICATION NUMBER

DATE OF FILING  
(day, month, year)PRIORITY  
CLAIMED

FRANCE

99 07181

6/8/99

YES X NO

YES NO

I hereby appoint the following attorneys and agent(s) to prosecute said application and to transact all business in the Patent and Trademark Office connected therewith and to file, prosecute and to transact all business in connection with international applications directed to said invention:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Figure 6

